

Induction of Cyclooxygenase-2 Accounts for Restraint Stress-Induced Oxidative Status in Rat Brain

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Cyclooxygenase (COX) is the rate-limiting enzyme in the metabolism of arachidonic acid into prostanoids. Although it is constitutively expressed in brain neurons, the inducible isoform (COX-2) is also upregulated in pathological conditions such as seizures, ischemia or some degenerative diseases. To assess whether COX-2 is regulated after stress, we have used adult male Wistar rats, some of which were immobilized during 6 h. An increase in PGE₂ concentration occurs in brain cortex after 2–6 h of the onset of stress as well as an enhancement of COX-2 protein. Immunohistochemical studies indicate that COX-2 is expressed in the cortex and hippocampus after stress in cells with morphology of neurons. Administration of PDTC (150 mg/kg), an inhibitor of the transcription factor NF- κ B or MK-801 (0.2 mg/kg), an N-methyl-D-aspartate receptor blocker, prevents both stress-induced increase in COX-2 activity and protein levels, suggesting an implication of these factors in the mechanism by which stress induces COX-2 in brain. To assess if COX-2 accounts for the oxidative status seen in brain after stress, a group of animals were i.p. injected with NS-398, a specific COX-2 inhibitor 1 h prior to the onset of stress. NS-398 (5 mg/kg) decreases stress-induced malondialdehyde accumulation in cortex as well as prevents the stress-induced oxidation of glutathione. Finally, NS-398 reduced Ca²⁺-independent inducible nitric oxide synthase (iNOS, NOS-2) activity and lowered the stress-induced accumulation of NO metabolite levels in cortex. These effects of NS-398 seem to be due to the specific inhibition of COX-2, since it has no effect on stress-induced corticosterone release, glutamate release, and NF- κ B activation. These findings are discussed as possible damaging and/or adaptive roles for stress-induced COX-2 in the brain.

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INTRODUCTION

The impact of stressor exposure on brain function has been widely investigated in the last years. Indeed, several reports indicate that long-lasting stress affects synaptic plasticity, dendritic morphology and neurogenesis in animals (Kim and Yoon, 1998), and induces both clinical and anatomical features of neurotoxic damage in humans (ie post-traumatic stress disorder) (Bremner *et al*, 1995). The main clinical correlates of stress-related neuropsychiatric disorders are depressive disorders, which are among the most common neuropsychiatric illnesses and represent a significant public health problem. Moreover, the relation between stressful events and the onset of disease (mainly cardiovascular and neurologic) is well documented (Baum and Posluszny, 1999).

The precise molecular and cellular events induced by stress and responsible for the brain damage found in this condition are still a matter of debate. The high-output isoform of nitric oxide (NO) synthase (NOS-2) has been implicated in cellular toxicity in many cell systems, including the brain (for a review, see Gross and Wolin, 1995). In this context, we have demonstrated that chronic restraint stress induces the expression of NOS-2 in rat brain cortex and hippocampus and that its inhibition protects against stress-induced cell damage in this model (Olivenza *et al*, 2000). Once NOS-2 is expressed, the formation of large amounts of oxygen and nitrogen reactive species may account for the oxidation of cellular components found after chronic restraint stress in the rat brain (Liu *et al*, 1996; Madrigal *et al*, 2001b). A plausible consequence of this is the volume reduction that has been described in these two brain structures in chronically depressed patients and other stress-related disorders (Sheline *et al*, 1996; Koenen *et al*, 2001; Cotter *et al*, 2002).

In order to further investigate other possible sources of oxidative status in the brain, we decided to focus on the cyclooxygenase (COX) pathway. COX (also referred to as prostaglandin endoperoxide synthase) catalyzes the first steps of the synthesis of prostanoids by converting

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arachidonic acid into prostaglandin H_2 , which is the common substrate for specific prostaglandin synthases. The enzyme is bifunctional, with fatty acid COX and hydroperoxidase activities. COX exists as two distinct isoforms (Fu *et al*, 1990; Xie *et al*, 1991): whereas COX-1 is constitutively expressed as a housekeeping enzyme in nearly all tissues, mediating physiological responses, COX-2 is rapidly and transiently induced in cells that are involved in inflammation and is primarily responsible for the synthesis of prostanoids involved in pathological inflammatory processes. COX-2 is constitutively expressed in only a few tissues, for example, the brain, but is upregulated in a wide variety of cells by cytokines, mitogens, growth factors, and bacterial lipopolysaccharides. In the brain, COX-2 is induced in pathological conditions such as seizure, ischemia, or other neurodegenerative diseases (Yamagata *et al*, 1993; Nogawa *et al*, 1998), and has been involved in the damage associated in these conditions (reviewed in Pasinetti, 2001). According to its condition of inducible enzyme, and similarly to NOS-2, the promoter of the immediate-early gene COX-2 depends on the activation of several transcription factors including NF- κ B (Appleby *et al*, 1994).

By using a stress acute paradigm that has been reported to cause neuronal damage after chronic exposure (Magariños and McEwen, 1995), we investigated if COX-2 was involved in this process and whether some of the effects of acute stress on the brain can be regulated by this pathway, as well as the possible relation with the expression of NOS-2. In fact, the relation between these two sources of oxidative status has been described in many *in vitro* and *in vivo* systems after exposure to inflammatory mediators such as cytokines and endotoxin (LPS) (Salvemini *et al*, 1993, 1994). Thus, the purpose of the present study was to evaluate the role of COX-2 as one of the mechanisms through which stress may lead to cellular oxidative status in the brain and the elucidation of its mechanisms of induction, which may suggest possible neuroprotective pharmacological manœuvres after stress.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 225–250 g were used. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense following Spanish and European rules (DC 86/609/CEE, RD 223/1988, and OM 13/X/1989). The rats were housed individually under standard conditions of temperature and humidity and a 12 h light/dark cycle (lights on at 08.00 am) with free access to food and water. All animals were maintained under constant conditions for 4 days prior to stress.

Restraint Stress

Rats were exposed to stress between 09:00 and 15:00 in the animal homeroom. The restraint was performed using a plastic rodent restrainer (Decapi-cone[®], Braintree, MA) that allowed for a close fit to rats during 6 h (Magariños *et al*, 1997; Leza *et al*, 1998) in their home cages. Control animals

were not subjected to stress, but were handled at 9:00. Animals were killed immediately after restraint (still in the restrainer) using sodium pentobarbital. Blood for plasma determinations was collected by cardiac puncture and anticoagulated in the presence of trisodium citrate (3.15% w:v, 1 vol citrate per 9 vol blood). After decapitation, the brain was removed from the skull and both cortical areas and other structures were excised from the brain.

COX Activity

PGE₂ and PGD₂ are the predominant PG species in the brain, and PGE₂ is most widely used as a marker of COX activity in neurons—specially in the cortex (Bishai and Coceani, 1992). Samples were purified using Amprep[™] (RPN1900 Amersham, Buckinghamshire, UK) minicolumns. After homogenizing cortices in the same buffer used for NOS activity determination (see below), the proper fraction needed for EIA isolation and PGE₂ quantification were carried out following the manufacturer's instructions (PGE₂ EIA, Amersham).

Characterization of COX-2, p65, and NOS-2 by Western Blot

To determine the levels of COX-2 and NOS-2, tissues were homogenized in the same buffer used for NOS activity (see below); for p65, cytosolic and nuclear extracts were obtained (see below), and after centrifugation in a micro-centrifuge for 15 min, the proteins present in the supernatant were loaded (20 μ g) and size-separated in 10% SDS-polyacrylamide gel electrophoresis (90 mA). The gels were processed against the A_gs and, after blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), were incubated with a specific goat polyclonal COX-2, rabbit polyclonal p65, and rat polyclonal NOS-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; all three at 1:1000 dilution). Proteins recognized by the antibody were visualized on X-ray film by chemiluminescence (ECL) following the manufacturer's instructions (Amersham Ibérica, Madrid, Spain). Autoradiographs were quantified by densitometry (Software Total Lab. Dynamics Ltd Phoretix, Newcastle, UK), and several time expositions were analyzed to ensure the linearity of the band intensities.

Immunohistochemistry

Rats were anaesthetized with sodium pentobarbital and perfused through the left ventricle with 100 ml of saline as a vascular rinse followed by 500 ml of fixative solution containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. The brains were removed, postfixed for 3 h in the same solution of paraformaldehyde at room temperature, and then cryoprotected by immersion overnight at 4°C in 0.1 M PB containing 30% sucrose.

Brains were frozen and serial 40- μ m-thick frontal sections were cut with a Leitz sledge microtome. Free-floating sections of the whole brain were incubated with specific antiserum and processed by the avidin-biotin peroxidase complex (ABC) procedure (Guesdon *et al*, 1978; Hsu and Raine, 1981; Rodrigo *et al*, 1994) to visualize immunoreactive sites for COX-2. All the free-floating sections were

incubated for 30 min in phosphate-buffered saline (PBS) containing 3% normal goat serum (ICN Biochemicals, Barcelona, Spain) and 0.2% Triton X-100, and then in COX-2 diluted 1:2500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS/Triton X-100 overnight at 4°C. After several washes in PBS, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin for 1 h. After washing, the sections were incubated with peroxidase-linked ABC (Vector Laboratories, Burlingame, CA, USA) for 90 min. The peroxidase activity was demonstrated by the nickel-enhanced diaminobenzidine procedure (Rodrigo *et al*, 1994).

Preparation of Cytosolic and Nuclear Extracts

A modified procedure based on the method of Schreiber *et al* (1989) was used. Tissues (brain cortex) were homogenized with 300 µl of buffer A (10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 0.1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml TLCK, 5 mM NaF, 1 mM NaVO₄, 0.5 M sucrose, and 10 mM Na₂MoO₄). After 15 min, Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s and nuclei were collected by centrifugation at 8000g for 5 min. Supernatants were taken as a cytosolic fraction. The pellets were resuspended in 100 µl of buffer A supplemented with 20% glycerol and 0.4 M KCl, and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13 000g for 5 min, and aliquots of the supernatant were stored at -80°C. All steps of the fractionation were carried out at 4°C.

Electrophoretic Mobility Shift Assay (EMSA) for NF-κB

Oligonucleotides were synthesized in an oligonucleotide synthesizer (Amersham Pharmacia Biotech, Little Chalfont, UK). The oligonucleotide sequence corresponding to the consensus NF-κB binding site (nucleotides -978 to -952) of the murine NOS-2 promoter was 5'TGCTAGGGGA TTTCCCTCTCTGT3' (Xie *et al*, 1994). Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme (Amersham, Spain) fragment in the presence of 50 µCi of [α -³²P]dCTP (Amersham, Spain) and the other unlabeled dNTPs in a final volume of 50 µl. DNA probe (5 × 10⁴ dpm) was used for each binding assay of nuclear extracts as follows: 15 µg of protein were incubated for 15 min at 4°C with DNA and 2 µg of poly(dI:dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10 mM Tris-HCl, pH 7.8, in a final volume of 20 µl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris borate-EDTA buffer (Díaz-Guerra *et al*, 1996). Supershift assays were carried out after incubation of the nuclear extracts with the antibodies against c-Rel proteins (0.5 µg) for 1 h at 4°C, followed by EMSA (anti-p65 and anti-p-50 from Santa Cruz Biotechnology, Santa Cruz, CA). The amount of radioactivity was quantified in a Fuji BAS 1000 radioactivity detection system, and the gels were exposed to X-ray films to obtain high-resolution images. In experiments using

X-ray films (hyperfilm), different exposure times were employed to ensure that bands were not saturated.

Lipid Peroxidation

This was measured by the thiobarbituric acid test for malondialdehyde (MDA) following the method described by Das and Ratty (1987) with some modifications. Half forebrain was homogenized in 10 vol 50 mM PB and deproteinized with 40% trichloroacetic acid (TCA) and 5 M HCl, followed by the addition of 2% (w/v) thiobarbituric acid in 0.5 M NaOH. The reaction mixture was heated in a water bath at 90°C for 15 min and centrifuged at 12 000g for 10 min. The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer. The results were expressed as nmol per mg of protein basis.

Brain Glutathione

These levels were measured as previously described (Anderson, 1985). The half forebrain was removed from the skull and frozen in ethanol. After that, tissues were rinsed in water and homogenized in 5% w/v 5-sulfosalicylic acid and centrifuged at 4500 g for 5 min. The supernatants were stored at 4°C until assayed. This supernatant is oxidized by 6 mM 5,5'-dithio-bis-(2-nitrobenzoic) acid (DTNB) to give reduced glutathione (GSSG) with the stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to glutathione (GSH) by the addition of highly specific glutathione reductase (266 U/ml from stock enzyme with 143 mM sodium phosphate and 6.3 mM Na₄ EDTA at pH 7.5) and 0.3 M NADPH. The rate of TNB formation is measured at 412 nm in a Beckman DU-7500 spectrophotometer and is proportional to the sum of GSH and GSSG present. Results were expressed as oxidized GSH per mg protein.

NO Synthase Activity

Cortices were snap-frozen in liquid nitrogen and stored until assayed at -80°C. Frozen tissues were homogenized by sonication (VibraCell) in an ice-cold buffer (pH 7.4) containing Tris-HCl (50 mM), sucrose (320 mM), dithiothreitol (1 mM), leupeptin (10 µg/ml) soybean trypsin inhibitor (10 µg/ml), and aprotinin (2 µg/ml), followed by centrifugation at 10 000g for 20 min at 4°C. NOS activity was determined in cell extracts under conditions (substrate and calcium concentration) of maximal activity to assess indirectly the amount of enzyme. The samples (40 µl of supernatant) were incubated at 37°C for 10 min in a buffer (KH₂PO₄ (50 mM), MgCl₂ (1 mM), CaCl₂ (0.2 mM), L-valine (50 mM), L-citrulline (1 mM), L-arginine (20 µM), and dithiothreitol (1.5 mM)) containing L-[¹⁴C]arginine (0.1 mCi/ml, Amersham Ibérica, Spain). The reaction was terminated by removing the substrate by the addition of 1 ml of 1:1 H₂O Dowex AF 500W-8 resin (Bio Rad). The activity of the calcium-dependent NOS was calculated from the difference between L-[¹⁴C]citrulline produced from control samples and samples containing ethylene glycol-bis(aminoethyl ether) N,N,N'-tetraacetic acid (EGTA, 1 mM); the activity of the calcium-independent isoform was determined from the difference between samples with

EGTA and samples containing 1 mM N^G -monomethyl-L-arginine (L-NMMA). The [^{14}C]-bound radioactivity was counted using a liquid scintillation counter (Beckman LS-6500) (Salter *et al*, 1991; Rees *et al*, 1995).

Brain NO_x^- (NO_2^- and NO_3^-) Levels

NO production was estimated from the amounts of its stable metabolites nitrite (NO_2^-) and nitrate (NO_3^-) in brain tissue. It was calculated by first reducing NO_3^- into NO_2^- in the presence of Cd (Cortas and Wakid, 1990) and NO_2^- was determined by a colorimetric assay based on the Griess reaction (Green *et al*, 1982) in a Thermomax[®] microplate reader (Molecular Devices). The measurement of NO_x^- levels has been found to be a reliable technique to determine the synthesizing capacity of NOS in the brain (Salter *et al*, 1996).

Plasma Corticosterone Levels

Plasma was obtained within 1 h of obtaining blood samples by centrifuging the sample at $\approx 1000g$ for 15 min immediately after stress. All plasma samples were stored at -20°C before assay by using a commercially available kit by RIA of ^{125}I -labeled rat corticosterone (DPC, Los Angeles, CA, USA). A gamma counter was used to measure radioactivity of the samples. The values obtained in control animals (198.30 ± 41.59 ng/ml) match with the kit manufacturer's expected values in adult male Wistar rats at the time of blood extraction ($\approx 15:00$ h).

Plasma Glutamate Concentration

Analysis of glutamate in plasma was performed by HPLC with fluorimetric detection (Perkin-Elmer Binary LC Pump 250 and Fluorescence Detector LC 240) (Lindroth and Mopper, 1979).

Pharmacological Tools

Various groups of animals were i.p. injected with a range (0.5–10 mg/kg) of NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide, a selective COX-2 inhibitor; Futaki *et al*, 1994) 1 h before restraint. NS-398 was dissolved in ethanol following the manufacturer's instructions. Selected groups of rats received PDTC (pyrrolidine dithiocarbamate, an inhibitor of the activation of NF- κ B; Schreck *et al*, 1992) or MK-801 ([(+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo (*a,d*) cyclohepten-5,10-imine]; dizocilpine), a specific noncompetitive *N*-methyl-D-aspartate (NMDA) antagonist (Wong *et al*, 1988). PDTC and MK-801 were administered at the onset of the stress and at doses used to reduce oxidative status in previous papers using this model (Madrigal *et al*, 2001a). Each experimental group contains six to eight animals.

Protein Assay

Protein assay Proteins were measured using bicinchoninic acid (Hill and Straka, 1988).

Chemicals and Statistical Analyses

Unless otherwise stated, the chemicals were from Sigma or RBI-Sigma (Spain). Data in text and figures are expressed as mean \pm SEM of the indicated number of experiments. Two group comparisons were evaluated by the paired or unpaired *t*-test, as appropriate. Multiple comparisons were analyzed by the ANOVA and Newman-Keuls test and $P < 0.05$ was considered as statistically significant.

RESULTS

COX-2 Activity and Expression in the Stressed Brain

We studied the enzymatic activity of COX-2 by determining the levels of the main brain COX-2 reaction product PGE_2 after restraint. Stress increased cortical PGE_2 levels after 2, 4, and 6 h ($P < 0.05$ vs control, 27.8 ± 2.6 ng/g protein, Figure 1a), and pretreatment with the selective COX-2 inhibitor NS-398 (0.5–5 mg/kg) attenuated significantly the stress-induced elevation of PGE_2 after 6 h (Figure 1a). The 72 kDa band corresponding to COX-2 protein increased at 4–6 h after the onset of stress as assessed by Western blot analysis (Figure 1b).

Concomitantly, we studied the localization of COX-2 in the brain. In control rats, few numbers of COX-2

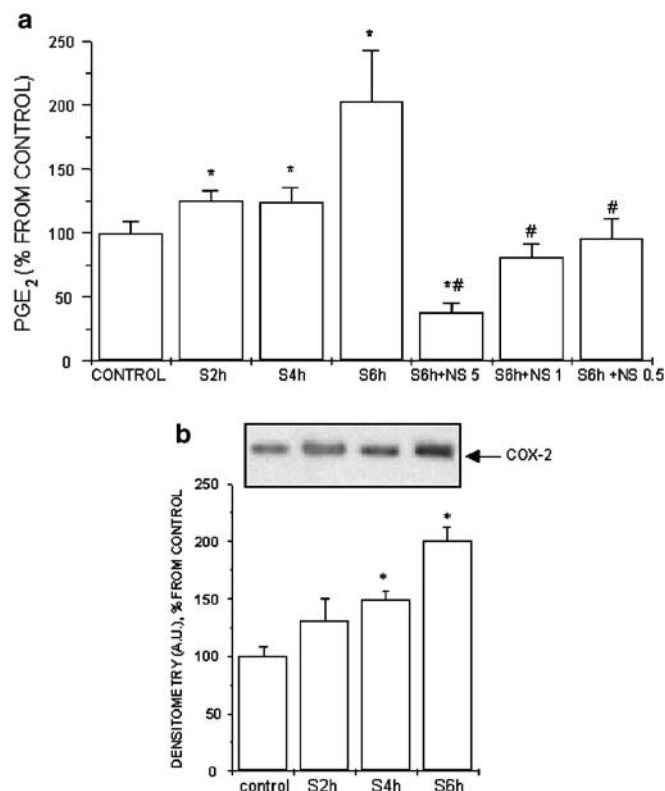


Figure 1 (a) Effect of restraint stress on PGE_2 in the brain cortex after 2, 4, and 6 h of stress (S2h, S4h, S6h). Effect of NS-398 (5, 1, and 0.5 mg/kg). PGE_2 in control: 27.85 ± 2.63 ng/g protein. The data represent the mean \pm SEM of six to eight rats. * $P < 0.05$ vs control; # $P < 0.05$ vs S6h (Newman-Keuls test). (b) Detection of COX-2 protein by Western blot (band: 72 kDa) and laser densitometric analysis of the band in the cortex of control and stressed rats by restraint during 2, 4, and 6 h (S2h, S4h, S6h). The data are representative of three experiments. * $P < 0.05$ vs control; # $P < 0.05$ vs S6h (Newman-Keuls test).

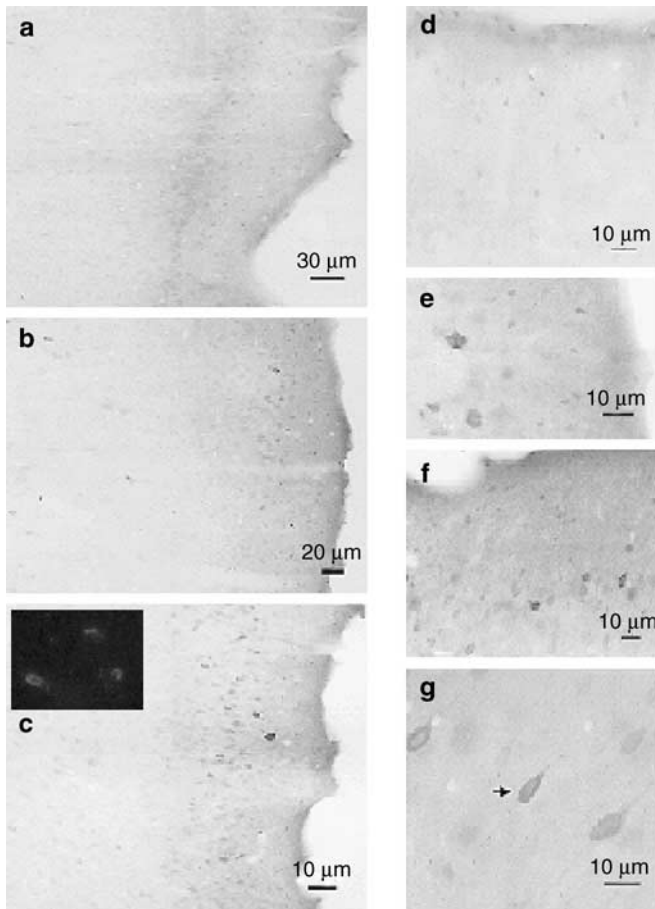


Figure 2 Comparative COX-2 staining and distribution of neuronal cells in sections from normal rat cingulate cortex (a and d) exposed to restraint stress for 4 h (b and e) and after 6 h (c, f, and g). COX-2 expression increases significantly in layers II–III and V–VI of the cingulate cortex (after 4 h of stress (b) and after 6 h of stress (c)). COX-2 expression was found in pyramidal neurons (g, arrow) and neurons with fusiform or typical multipolar morphology as associative neurons. Inset: COX-2 positive neurons (Cy5—labeled) in the fronto-parietal cortex after 6 h of restraint stress. Scale bars: (a), (b), and (c), 20 μm; (d)–(g) and inset, 10 μm.

immunoreactive neurons were observed in selected brain cortical areas. The cingulate and frontal cortices show immunoreactive neurons in layers II–III and V–VI (Figure 2a, d). Occasional and scattered immunoreactive neurons were distributed through parietal, temporal, insular, and pyriform cortices. An increase in COX-2 immunoreactivity was observed after 4 h (Figure 2b, e) and 6 h (Figure 2c–g and inset) of the onset of stress. These neurons have normal morphological characteristics, with a large unstained round nucleus. The immunoreactivity was characteristically perinuclear as previously described (Yamagata *et al*, 1993). In control rats few COX-2 positive neurons were observed in some hippocampal, hypothalamic, and thalamic areas, increasing the number of immunoreactive neurons in these selected brain areas after 4 and 6 h (not shown).

Mechanisms of COX-2 Expression in Stress

To assess some possible mechanisms in COX-2 expression in stress, we studied the effects of the selective COX-2 inhibitor NS-398. NS-398 inhibited both stress-induced and

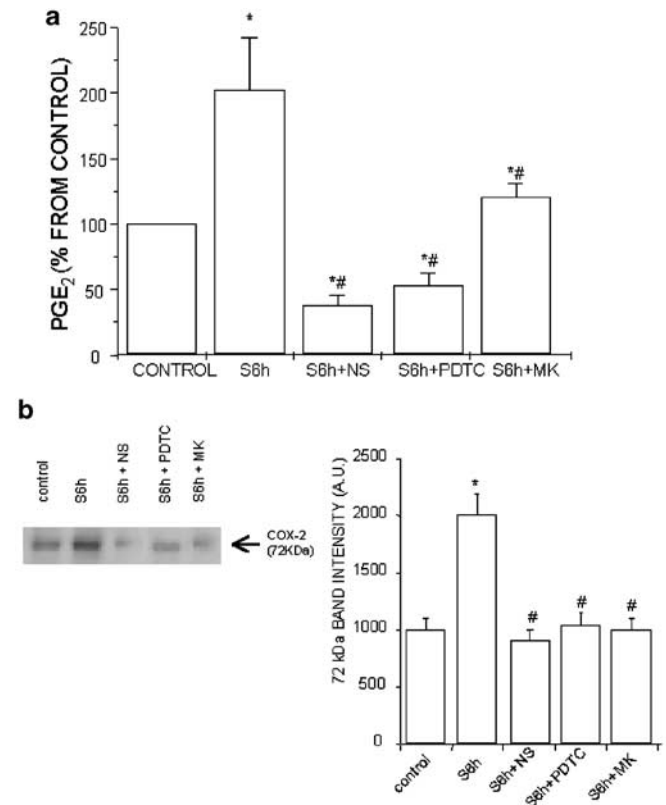


Figure 3 (a) Effect of restraint stress on PGE₂ in the brain cortex after 6 h of stress (S6h) and stressed rats treated with NS-398 5 mg/kg (S6h+NS), PDTC 150 mg/kg (S6h+PDTC), and MK-801 0.2 mg/kg (S6h+MK). PGE₂ in control: 27.85 ± 2.63 ng/g protein. The data represent the mean \pm SEM of six to eight rats. * $P < 0.05$ vs control; # $P < 0.05$ vs S6h (Newman–Keuls test). (b) Detection of COX-2 protein by Western blot (band: 72 kDa, left panel) and laser densitometric analysis of the band (right panel) in the cortex of control and stressed rats by restraint during 6 h (S6h) and stressed rats treated with NS-398 5 mg/kg (S6h+NS), PDTC 150 mg/kg (S6h+PDTC), and MK-801 0.2 mg/kg (S6h+MK). * $P < 0.05$ vs control; # $P < 0.05$ vs S6h (Newman–Keuls test). The data represent the mean \pm SEM of six rats.

control levels of PGE₂ (Figure 3a), and also decreased the stress-induced COX-2 protein level after 6 h of stress (Figure 3b).

We then studied PGE₂ concentration in PDTC-treated rats to assess the possible relation between COX-2 and the activation of NF- κ B. Pretreatment with PDTC (150 mg/kg) reduced stress-induced increase in PGE₂ concentration in $26.1 \pm 4.6\%$, $P < 0.05$ vs S6h (Figure 3a). PDTC also reduced COX-2 expression at this time (Figure 3b).

Since it has been found that stimulation of glutamate receptors after excitatory amino acids (EAAs) released during stress leads to the activation of NF- κ B in neurons (Guerrini *et al*, 1995; Kaltschmidt *et al*, 1995), we decided to explore this possibility. The NMDA receptor blocker MK-801 (0.2 mg/kg) decreases both stress-induced PGE₂ accumulation and COX-2 expression (Figure 3a and b, respectively).

Effect of Stress-Induced COX-2 Activation in Oxidative Parameters

Restraint caused the accumulation of the mediator of lipid peroxidation MDA in cortex as soon as 6 h after stress

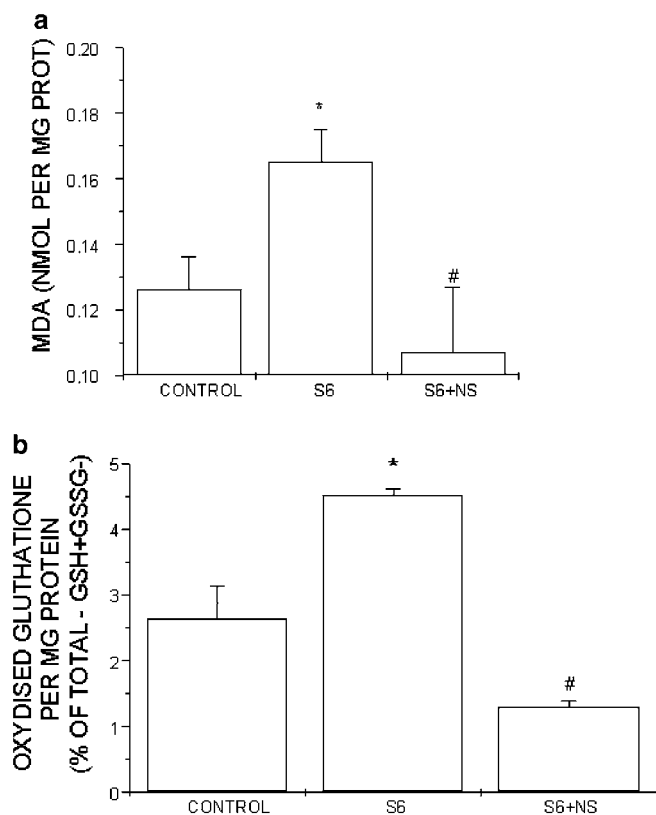


Figure 4 (a) Effect of acute restraint exposure on MDA levels in the brain cortex of control and stressed rats for 6 h (S6) with or without NS-398 5 mg/kg (S6+NS) 1 h before restraint. The data represent the means \pm SEM of six rats. * $P < 0.05$ vs control; # $P < 0.05$ vs S6 (Newman–Keuls test). (b) Effect of acute restraint exposure on oxidised glutathione levels in the brain cortex of control and stressed rats for 6 h (S6) with or without NS-398 5 mg/kg i.p. 1 h before restraint (S6+NS). The data represent the means \pm SEM of eight rats. * $P < 0.05$ vs. control; # $P < 0.05$ vs S6 (Newman–Keuls test).

exposure (Figure 4a). Concomitantly with the increase in lipid peroxidation markers, restraint produced an increase in the concentration of oxidised glutathione (Figure 4b). Administration of NS-398 (5 mg/kg i.p.) 1 h before the onset of stress prevented stress-induced increase in both parameters, MDA and oxidised glutathione (Figure 4a and b, respectively).

Relation Between Stress-Induced COX-2 and NOS-2 in Brain

The other powerful source of oxidative mediators, NOS-2, was expressed in the cortex after 6 h of stress exposure, as seen by Western blot analysis and activity studies (Figure 5a). This increase occurred concomitantly with an elevation in the stable metabolites of NO (NO_x^-) in brain tissue (Figure 5b). Pharmacological inhibition of COX-2 with NS-398 (5 mg/kg) prevented Ca^{2+} -independent NOS activity (Figure 5a) as well as NO_x^- accumulation (Figure 5b), but had no effect on NOS-2 protein (Figure 5a, inset).

To test the possibility that NS-398 acts via a direct mechanism on calcium-independent NOS activity, a citrulline assay was performed with cortical tissue from six rats injected with LPS (1 mg/kg i.p.) and killed after 24 h. NS-398 (10 $\mu\text{mol/l}$) did not have any direct effect on this activity

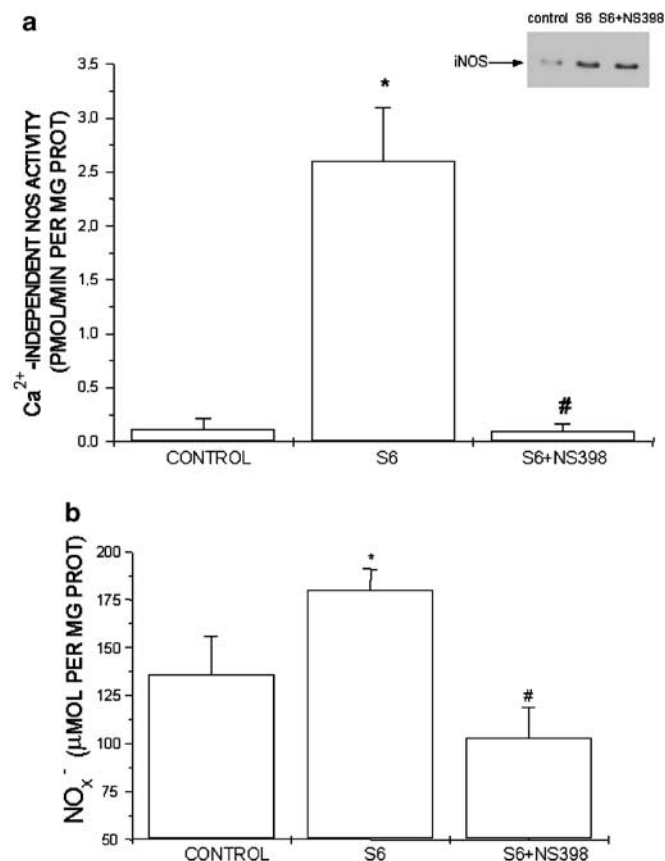


Figure 5 (a) Calcium-independent NOS activity in the cortex from control and stressed rats after 6 h of restraint (S6) and stressed rats receiving NS-398 5 mg/kg i.p. 1 h before restraint. Inset: detection of NOS-2 protein by Western blot (band: 133 kDa) in control and stressed rats by restraint 6 h with or without NS-398. The data represent the mean \pm SEM of six to eight rats. * $P < 0.05$ vs control; # $P < 0.05$ vs S6 (Newman–Keuls test). (b) Effect of restraint exposure on NO_2^- and NO_3^- (NO_x^-) levels in the brain cortex of control and stressed rats during 6 h (S6) with and without a dose of 5 mg/kg i.p. of NS-398 1 h before stress. The data represent the means \pm SEM of six rats. * $P < 0.05$ vs control; # $P < 0.05$ vs S6 (Newman–Keuls test).

(LPS: 29.03 ± 14.60 ; LPS + NS-398: 21.92 ± 12.86 pmol/min per mg protein, $P > 0.05$). NS-398 (10 mg/kg) did not modify basal levels of NO_x^- in brain tissue (control: 136 ± 24 ; NS-398: 125 ± 14 μmol per mg protein, $P > 0.05$).

Mechanisms of NS-398 Preventive Properties in Stress

Once the preventive effect of NS-398 in stress-induced oxidative status was established, we sought to study possible effects prior to COX-2 inhibition.

First, to test the possibility that the effects of selective COX-2 inhibition were related to an interference on the general response to stress, we determined plasma corticosterone levels. NS-398 (5 mg/kg) did not affect corticosterone increase after 6 h of restraint (control: 198.30 ± 40.59 ng/ml; stress 6 h: 392.15 ± 17.70 ng/ml, $P < 0.05$ vs control; stress 6 h plus NS-398: 428.55 ± 41.92 ng/ml, $n = 6$, $P > 0.05$ vs stress 6 h).

Second, we determined the effect of NS-398 on stress-induced increase in plasma levels of glutamate. NS-398 (5 mg/kg) failed to act through inhibition of glutamate

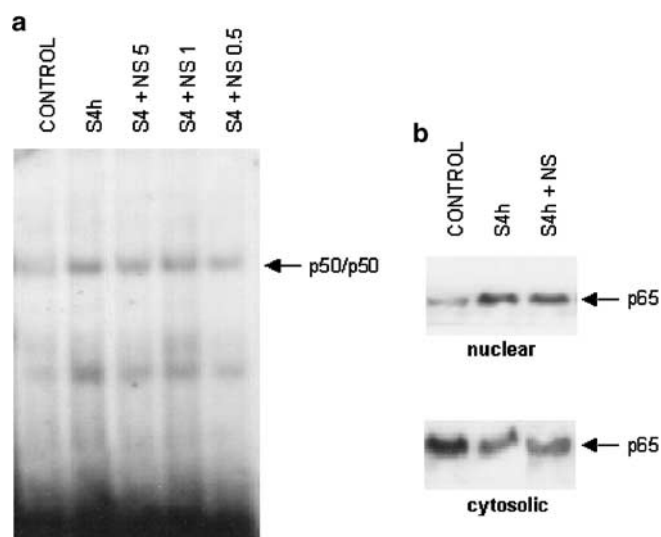


Figure 6 (a) NF- κ B translocation to the nucleus as measured by electrophoretic mobility shift assay (EMSA, left panel; see Materials and methods) of p50/p50 in nuclear extracts from the brain cortex of control and stressed rats during 4 h (S4h) with and without NS-398 (5, 1, or 0.5 mg/kg i.p.) 1 h before stress. The data are representative of six rats. No statistical significances were found (Newman–Keuls test) in densitometric analysis. (b) Western blot analysis of p65 in nuclear and cytosolic extracts from the brain cortex of control and stressed rats during 4 h (S4h) with and without a dose of 5 mg/kg i.p. of NS-398 1 h before stress. The data are representative of six rats. No statistical significances were found (Newman–Keuls test) in densitometric analysis.

release (control: 8266 ± 483 ; stress 6h: 9469.8 ± 416.9 ; S6 + NS 5 mg/kg: 8962 ± 172.5 ng glutamate/ml, $n = 6$, $P > 0.05$ vs control).

Third, to assess whether specific inhibition of COX-2 activity accounts for changes in NF- κ B function, we studied the effects of NS-398 in the activation of NF- κ B subunits. Interestingly, NS-398 did not modify either the stress-induced p50/p50 translocation to the nucleus as assessed by EMSA in nuclear extracts (Figure 6a) or the p65 activation as seen by the lack of changes in Western blot studies in cytosolic and nuclear fractions (Figure 6b).

DISCUSSION

The results of this study provide evidence that acute restraint stress induces the expression of COX-2 in rat brain. This conclusion is supported by Western blot, immunohistochemical, and enzymatic activity analyses. Furthermore, the finding of the effects of specific pharmacological inhibition of COX-2 preventing the increase on membrane lipid peroxidation mediators and the depletion of the main tissular antioxidant glutathione strongly suggest that this COX isoform is involved in the accumulation of oxidative mediators found in the brain in this particular experimental model. Finally, an interaction of COX-2 with NOS-2, another known source of oxidative mediators in the stressed brain, is presented in this paper. The effects of NS-398 seem to depend mainly on its COX-2 inhibitory properties, since it did not affect other mechanisms induced by stress such as corticosterone elevation, glutamate release, or NF- κ B activation.

It is well known that COX-1 is constitutively expressed in nearly all tissues including brain, in neurons and astrocytes (Hewett *et al*, 2000). COX-2, although an inducible protein, is constitutively expressed in only a few tissues including the brain, particularly in neuronal soma, not in astrocytes, of different regions including cortex, amygdala, hippocampal formation, and the dorsal horn of the spinal cord (Yamagata *et al*, 1993; Breder *et al*, 1995). It becomes upregulated briefly—after 1–8 h—after seizure, trauma, intracerebral hemorrhage, ischemia, or other neurodegenerative diseases (Yamagata *et al*, 1993; Beiche *et al*, 1996; Nogawa *et al*, 1998; Knott *et al*, 2000; Kim *et al*, 2001; Gong *et al*, 2001). Other types of brain cells, including microglia, astrocytes, and vascular cells, do not express significant levels of COX-2 normally, but its expression is also increased after cerebral insults (Laflamme *et al*, 1999).

After the initial observation by Yamagata *et al* (1993) of the increase in COX-2 mRNA in mouse cerebral cortex after swim stress, the data presented here are, to our knowledge, the first to demonstrate the localization of COX-2 immunoreactive neurons in cingulate and frontal cortex, as well as the relation between COX-2 expression and the oxidative status seen in this condition. In our model, the cerebral cortex seems to be the area in which changes in COX-2 are more evident at this time of stress. Although most of the information about the changes seen after stress refers to the hippocampus, the cerebral cortex seems to be one of the target areas of damage in animal models of stress-related disorders and in humans with major depression (McEwen, 2000; Cotter *et al*, 2002; Bremner *et al*, 2002). This is also the brain area with the highest PGE₂ content (the predominant PG species in the brain) (Abdel-Halim and Anggard, 1979).

The use of NS-398 as a tool to inhibit specifically COX-2 activity in the brain after peripheral administration has allowed us to conclude that the increase in activity and expression of this protein results in the production of an oxidative environment in the brain. The resulting membrane lipid peroxidation due to oxyradical attack on membrane fatty acids results in the formation of several cytotoxic aldehyde products such as MDA (Esterbauer *et al*, 1991). This process has been described to play an important role in the pathogenesis of several neurodegenerative diseases (reviewed in Mattson, 1998), and we and others have described the formation of large amounts of oxygen and nitrogen reactive species and subsequent lipid peroxidation during stress in the brain (Liu *et al*, 1996; Madrigal *et al*, 2001b), liver, and heart (Hu *et al*, 2000). Concomitantly, an increase in lipid peroxidation may also be due to an insufficiency of the protective antioxidant systems (mainly GSH) as we previously demonstrated to occur in chronic (21 days) and acute (6 h) restraint stress (Madrigal *et al*, 2001b; De Cristóbal *et al*, 2002). Thus, an important finding in the present study is the capacity of NS-398 to prevent the increase in brain oxidized GSH (~172 %) that occurs after 6 h of stress exposure.

We have shown that NS-398 inhibits NOS-2 activity and decreases NO $_{x}^{-}$ accumulation without any direct effect on the enzyme, providing functional evidence that the interaction between these two sources of oxidative damage occurs at the level of their catalytic activity. The interactions between iNOS and COX-2 pathways may lead to contro-

versal results depending on the cell system studied and the experimental conditions. In our model of restraint stress, we have shown that activation of COX-2 precedes NOS-2 probably because, although protein synthesis is required in both cases, COX-2 remains constitutively expressed in the brain (Yamagata *et al*, 1993). The relations between these two sources of oxidative status are consistent with the fact that, as observed also in brain ischemia, COX-2 is expressed before NOS-2 (6 and 12 h, respectively, after transient occlusion of the MCA) (Nogawa *et al*, 1998) and 30 min to 2 h after reperfusion (Domoki *et al*, 1999). On the other hand, these data strongly suggest that both enzymes work in the same direction to produce oxidative status in acute stress. Further studies will be addressed to assess their relation in chronic stress but, in principle, one possibility is that the accumulation of oxidative-nitrosative species, including the powerful oxidant peroxynitrite (ONOO⁻) seen after 21 days of repeated restraint (Madrigal *et al*, 2001b), came also from COX-2. Indeed, the two catalytic steps of COX-2, COX and peroxidase, are associated with the production of reactive oxygen species and, similarly to NOS-2, COX-2 could act as a source of superoxide for ONOO⁻ formation (Vane *et al*, 1998).

It remains to study the precise mechanisms responsible for COX-2 expression and activity in stress. It is well known that basic gene induction mechanisms, including increase in NF- κ B-DNA binding, is fundamental in COX-2 expression in neurodegenerative disorders (Lukiw and Bazan, 1998). Furthermore, known inhibitors of NF- κ B activation, including PDTC and PKC inhibitors, strongly reduced COX-2 transcription (Bauer *et al*, 1997; Callejas *et al*, 1999). The effect of PDTC decreasing stress-induced accumulation of PGE₂ and COX-2 protein described in this paper suggests the involvement of NF- κ B mechanisms. This might be an important mechanism since it has been demonstrated that NF- κ B is activated in the brain after acute stress and accounts for NOS-2 expression in this model (Madrigal *et al*, 2001a) contributing to the oxidative status observed in this condition. On the other hand, it is possible also that locally produced lipid peroxidation products mediate COX-2 activity and expression. Indeed, COX-2 may be, in turn, upregulated by mediators of oxidative stress such as oxidized fatty acid metabolites (4-hydroxy-2-nonenal, HNE) and MDA, as has been shown in rat liver epithelial (RL34) cells (Kumagai *et al*, 2000). This possibility could explain the decrease in COX-2 expression induced by NS-398 observed in this paper. The possibility that, in chronic stresses, such a feedback process takes place deserves further attention.

It is well known that glutamate and other EAAs, mainly through the NMDA receptor, have been implicated in the pathogenesis of stress-induced brain injury (Sapolsky *et al*, 1990; Moghaddam, 1993; Magariños and McEwen, 1995; Kim *et al*, 1996). Several studies have described increases in brain (McBride *et al*, 1976; Gilad *et al*, 1990; Moghaddam, 1993) and plasma EAA levels (Milakofsky *et al*, 1985; De Cristóbal *et al*, 2002) following exposure to restraint or immobilization. Although glutamate concentration in this report has been determined in plasma, its increase after stress very likely reflects the profile of this EAA in the brain, as has been shown to be the case after cerebral ischemia (Baker *et al*, 1995; Castillo *et al*, 1997; De Cristóbal *et al*, 2001). It is well known that one of the main mechanisms of

glutamate toxicity in neurodegenerative diseases is the oxidation of transporters which account for inhibition of EAA reuptake (Trotti *et al*, 1996). Although the present study focuses on changes that occurred after an acute unique stress session of 6 h, the fact that specific blockade of NMDA receptors prevented both COX-2 activity and expression could indicate a new neuroprotective mechanism of strategies minimizing excitotoxicity, although caution should be emphasized with the use of pharmacological tools blocking physiological NOS-1 production of NO.

Finally, after the description of the role of COX-2 in stress and its relations with mediators of cellular oxidative status in the brain, the effects of NS-398 described in this paper seem to be directly related to the inhibition of COX-2 activity. First, NS-398 did not interfere with the systemic response to stress, since it did not affect the stress-induced corticosterone increase. Second, the fact that pharmacological inhibition of COX-2 by NS-398 did not modify the stress induced plasma glutamate levels discard a role in the glutamatergic mechanisms. Third, NS-398 did not affect the activity of NF- κ B.

In conclusion, we have investigated the role of COX-2 after acute stress in the brain cortex. The increase in COX-2 expression and activity appears very early after the onset of stress, and accounts, at least in part, for the accumulation of oxidative mediators in this condition. A question of particular interest is the possibility that the changes seen would be a feature not of the deleterious effects of stress, but of some potentially reversible type of adaptive plasticity, as proposed by McEwen (1998). In this vein, the implication of constitutive COX-2 expression in the brain and its upregulation by stress could also be physiological (reviewed in Dubois *et al*, 1998), a question that deserves further consideration. In fact, a kind of 'oxidative stress' occurs in the normal brain (Reagan *et al*, 2000), and evidence for a physiological or pathophysiological role for lipid peroxidation has been presented (reviewed in Parola *et al*, 1999) in addition to its well-defined deleterious consequences (reviewed in Mattson, 1998).

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